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INCREASED CD95 (Fas) AND PD-1 EXPRESSION IN PERIPHERAL BLOOD T LYMPHOCYTES IN COVID-19 PATIENTS

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Running Title: CD95 and PD-1 expression in COVID-19 patients

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SUMMARY A low count of CD4+ and CD8+ lymphocytes is a hallmark laboratory finding in the Coronavirus disease 2019 (COVID-19). Using flow cytometry, we observed significantly higher CD95 (Fas) and PD-1 expression on both CD4+ T and CD8+ T cells in 42 COVID-19 patients when compared to controls. Higher CD95 expression in CD4+ cells correlated with lower CD4+ counts. A higher expression of CD95 in CD4+ and CD8+ lymphocytes correlated with a lower percentage of Naive events. Our results might suggest a shift to antigen-activated T cells, expressing molecules increasing their propensity to apoptosis and exhaustion during COVID-19 infection.

Key words: COVID-19, CD95 (Fas), PD-1, apoptosis, exhaustion

INTRODUCTION

Severe acute respiratory syndrome Cov-2 (SARS-CoV-2) has been identified as a novel genus-beta-coronavirus with an outbreak of unusual viral pneumonia in Wuhan, China, and that progressively became pandemic.[1] The clinical spectrum of Coronavirus disease 2019 (COVID-19) appears to be wide, from an asymptomatic infection or a mild upper respiratory tract illness in a large group of patients to a severe interstitial pneumonia with Acute Respiratory Distress Syndrome (ARDS) and even death. In symptomatic patients the disease is characterized by a marked increase of cytokines, such as IL-6, and high level of inflammatory parameters including C-reactive protein. Patients with the most severe clinical presentations are elderly and have co-morbidities.[2]

Lymphopenia has been described as a hallmark finding in the 2003 outbreak of coronavirus-associated SARS and is the most frequent hematological abnormality also in COVID-19 patients.[3] Baseline lymphocyte count was found to be lower in patients with COVID-19 critical illness and non-survivors. Of note, patients at higher risk of ARDS showed a lower count of CD4+ and CD8+ lymphocytes.[4] Moreover, the expression of exhaustion markers, as PD-1 and TIGIT, in CD8+ T cells was higher in patients with a severe clinical course.[5]

Fas (CD95), a cell surface receptor of the tumor necrosis factor superfamily, has long been viewed as a death receptor that mediates apoptosis to maintain immune homeostasis.[6] CD95 is widely expressed in Memory and Effector T cells upon contact with antigen, while Naive T cells are typically CD95 negative.[7,8]

Programmed cell death 1 (PD-1, CD279), an antigen of Effector T cells, is considered an exhaustion marker, also expressed during antigen-mediated T cell activation.[9] Upregulation of PD-1 is observed during acute infections and after infection with persistent virus, including HIV, HBV and HCV. In particular, PD-1 expression in HIV specific-CD4+ and CD8+ lymphocytes is associated with T-cell exhaustion and disease progression.[10]

In our study we assessed the expression of these surface markers, CD95 and PD-1, in peripheral blood T lymphocytes in COVID-19 patients, correlating also this phenotype with the T cell maturational pattern.

METHODS

Multiparametric flow cytometry was performed on EDTA-peripheral blood samples collected from 42 new consecutive cases of COVID-19 at admission to Fondazione Agostino Gemelli Academic Hospital. All cases were diagnosed by SARS-Cov-2 nucleic acid testing of throat swab specimens using RT-PCR. Informed consents were obtained for all patients. The study protocol was approved by the Ethical Committee of the Fondazione Policlinico Agostino Gemelli IRCCS (protocol number 0017456/20).

The percentages and absolute counts of CD3+, CD4+, CD8+ lymphocytes were obtained with a single-platform method using a standard antibody cocktail TETRA-1 by AQUIOS cytometer (Beckman Coulter). We next performed an extracellular staining with the following monoclonal antibodies: CD45RAFITC, CCR7-PE, CD3-PerCP-Cy5.5, CD95-APC, CD4-APCH7, PD-1-BV450, CD45-BV500 (BD Biosciences) and CD8-PECy7 (Beckman Coulter). Data were acquired with FACSCanto II cytometer and analyzed with FACSDiva Software (BD Biosciences). A minimum of 30000 CD3+ events per tube were recorded. The CD4+ and CD8+ T cells were selected among CD3+ population. The percentages of CD95 and PD-1 expression were analyzed in CD4+ and CD8+ cells. To set the gate on positive CD95 or PD-1 events we used a negative control tube lacking antibodies against CD95 and PD-1. CD4+ and CD8+ T cell maturational subsets were defined as: Naïve (CD45RA+CCR7+), Central Memory (CM; CD45RA-CCR7+), Effector Memory (EM; CD45RA-CCR7-), Terminal Effector Memory (TEMRA; CD45RA+CCR7-).

Association between parametric continuous variables was performed by Pearson correlation. The Wilcoxon-Mann-Whitney was used for two-sample comparisons (controls-patients). Data were summarized as medians and ranges. *P*-values < 0.05 were considered statistically significant. Statistics were carried out with the NCSS10 Software.

RESULTS

We studied 42 patients with a wide age distribution range (median age 73, range 29-93); 46% were males. The most frequent symptoms were fever, dyspnea and cough. Median time from symptoms onset to sample collection was 4 days (range 1-10). No patients had been treated before blood sampling nor had recently received corticosteroids. None had concomitant viral infections (HIV, HBV, HCV). All patients were hospitalized and five were subsequently transferred to intensive care unit (ICU) (median age 71, range 62-87); one ICU and five non-ICU patients died (median age 83, range 71-93). As lymphocyte phenotypes may be influenced by immunosenescence we divided patients into two groups: < 65 years (median age 56, range 29-65, n=15) and $\geq$ 65 years (median age 82, range 67-93, n=27). We compared the younger patients both to an age-matched group of 19 healthy controls and to the older patients (median age 56, range 28-65). We also compared the older patients to an age-matched group of 20 inpatients (median age 79, range 67-92) with no clinical history of cancer, infections, rheumatological diseases.

According to previous reports CD4+ and CD8+ absolute counts were significantly lower in young patients than in healthy controls and comparable between young and old patients. The CD4/CD8 ratio was not inverted in patients (Table 1).[3,4] In CD4+ T cells CD95 expression was significantly higher in patients than in controls and comparable between young and old patients (Fig.1, Table 1)**.** Moreover, higher CD95 expression correlated with lower CD4+ absolute count (*R* 0.14, p=0.02). In CD8+ T cells we observed a homogeneous and higher CD95 expression in patients compared to a heterogeneous expression in controls (Fig.1, Table 1). In patients this expression increased with age (*R* 0.28, p=0.0003).

PD-1 expression in CD4+ and CD8+ T lymphocytes was significantly increased in **young** patients than in controls, with a comparable expression between young and old patients (Fig. 1, Table 1). Specifically, PD-1 was expressed in CD95+ lymphocytes and a positive correlation between CD95 and PD-1 percentages was observed among CD4+ cells (*R* 0.28, p=0.02).

Likewise, significant lower counts of CD3+, CD4+, CD8+ lymphocytes and higher CD95 and PD-1 expression were observed in old patients compared to inpatients, with a trend for PD-1 expression in CD8+ cells (p=0.065) (Table 1).

In a subgroup of 16 patients (median age 61, range 29-91) we analyzed the T cell maturation pattern. In CD4+ lymphocytes a higher CD95 expression correlated both with a lower percentage of Naïve (Fig.1) and with a higher percentage of EM cells (*R* 0.70, p<0.00001 and *R* 0.82, p<0.00001, respectively). In CD8+ T cells a higher CD95 expression correlated with a lower percentage of Naïve (*R* 0.81, p=0.00014) (Fig.1) and with a higher percentage of TEMRA cells (*R* 0.65, p=0.00631). No correlation was observed between T cell maturational subsets and PD-1 expression.

Finally, in ten patients developing severe illness (ICU and/or death) compared to others we observed a significant lower CD4+ absolute count (median value $202x10^6$/L, range 66-731, versus $507x10^6$/L, range 104-1316, p=0.01) and a higher CD95 expression (median value 80%, range 54-92, versus 68%, range 44-92, p= 0.02).

DISCUSSION

In 42 Caucasian COVID-19 patients, we confirmed a significant lymphopenia and reduction of CD4+ and CD8+ T cells at the time of hospital admission both in young and old patients. CD4+ and CD8+ lymphopenia has been described as an unfavorable prognostic factor.[2,4] Therefore, we can suppose that T lymphopenia in COVID-19 infection could be a negative prognostic factor independently from age. Furthermore, we report an increased CD95 and PD-1 expression in circulating CD4+ and CD8+ lymphocytes in patients, regardless of age. Both antigens are known to be upregulated upon T cell activation, and can signal a propensity for apoptosis (CD95) or T cell exhaustion (PD-1). We observed an increased CD95 expression in CD8+ T cells with older age according to previously described higher susceptibility to CD95-induced apoptosis in elderly individuals.[11] The upregulation of CD95 in CD4+ T cells combined with CD4+ lymphopenia has been reported during other viral infections

as HIV, respiratory syncytial virus and measles.[12-13-14] In our patients we observed a direct correlation between CD95 expression and a lower CD4+ absolute count, suggesting a comparable mechanism. Our data on increased PD-1 expression is in line with data by Zheng et al showing an exhausted T cell phenotype in patients with severe COVID-19 infection.[5]

The increased CD95 and PD-1 expression in T lymphocytes in COVID-19 might suggest a shift from Naïve T cells to Memory T cells. This hypothesis can be supported by the observation of an inverse correlation between percentage of CD95 expression and percentage of Naïve population both in CD4+ and CD8+ T cells. Moreover, CD95 expression directly correlated with a Memory phenotype, in particular Effector Memory in CD4+ and Terminal Effector Memory in CD8+ lymphocytes.

As previously described, T-cell adaptive immune responses are necessary to temper the early over-activation of the innate immune response.[15] Our results suggest that reduced numbers and potentially impaired functional status of the adaptive immune response might contribute to the over-activation of innate immune response and to the severity of COVID-19 infection.

More clinical data as well as a larger number of patients are needed to assess the clinical impact of our findings to better understand the functional and prognostic role of PD-1 and CD95 in COVID-19 illness. Determination of cytokines involved in the regulation of immune response, as IL-6, TNF-alfa, IL-10, IL-2, might also be helpful. Longitudinal assessment of phenotypic characteristics throughout treatment might allow to monitor for disease evolution and treatment response.

In conclusion, apoptosis via CD95 could be a possible mechanism for COVID-19 induced lymphopenia and our data provides new insights into the functional competence of T lymphocytes in COVID-19 infection.

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Authors contributions SB, EMe, PC, SH, VDS and EMa designed the research study, performed research, analyzed data and wrote paper. FM, SD, MLS, MF performed flow cytometric analysis.

MF, RM, AC, SS, AG, MS, and FR contributed patients and were involved in critical revision of the report. All authors approved the final manuscript.

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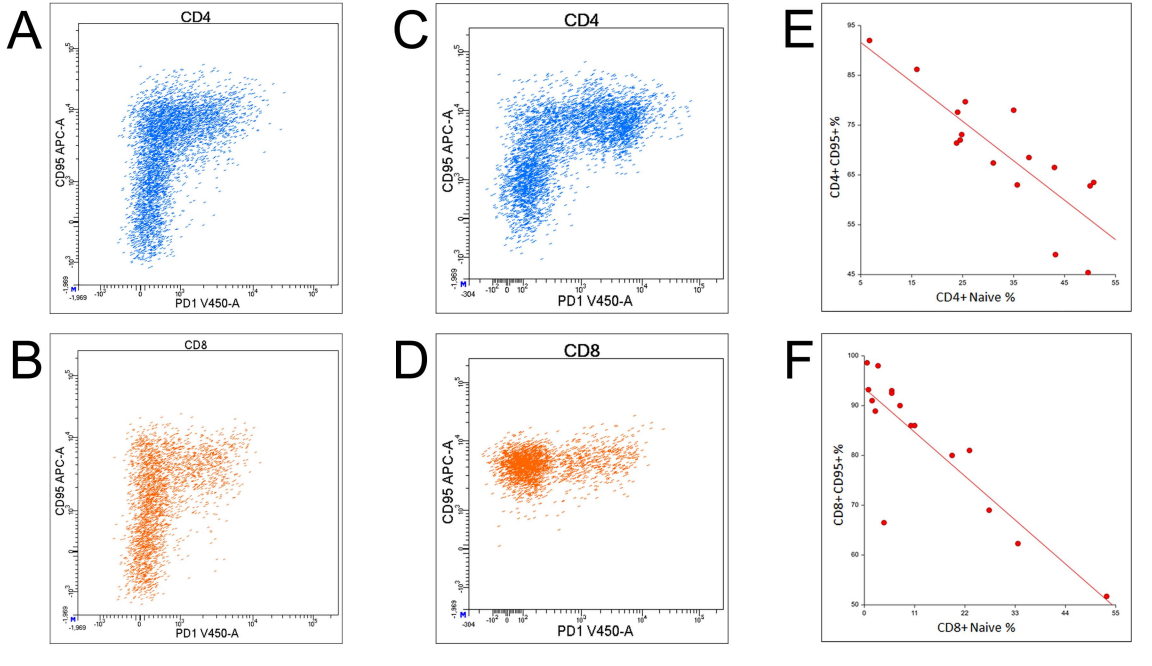
Supporting Information

Figure 1. Expression of CD95 and PD-1 in CD4+ and CD8+ circulating T lymphocytes in one healthy control (A and C) and one COVID-19 patient (B and D). Correlation between the percentage of CD95 expression and the percentage of Naïve CD4+ (E) and CD8+ (F) circulating T lymphocytes.


A
CD4
CD95 APC-A
PD1 V450-A
B
CD8
CD95 APC-A
PD1 V450-A
C
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CD95 APC-A
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